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3

4 Short communication

5 **Purified natural pig immunoglobulins can substitute dietary zinc in**
6 **reducing piglet post weaning diarrhoea**

7

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15

16

17 **Abstract**

18 Enteric infectious disease in weaner piglets, including postweaning diarrhoea (PWD), are
19 usually treated and/or prevented with antibiotics and/or zinc oxide in the piglet feed.

20 However extensive use of antibiotics and zinc oxide in intensive animal production is
21 unwanted as it may promote microbial antibiotic resistance and pose environmental
22 problems. Recently, in an experimental model of PWD, we observed that oral
23 administration of purified porcine immunoglobulin G (pplgG) from pooled natural pig
24 plasma could reduce enteric infection. In the present study we were able to reproduce
25 these results as it was observed that oral pplgG accelerated clearance of faecal
26 haemolytic bacteria in pigs challenged with *E. coli* in comparison with pigs not receiving
27 pplgG. This effect was observed upon feeding pplgG for seven days postweaning
28 suggesting that pplgG does not have to be used prophylactically for several days
29 preweaning. Furthermore, the effect of oral administration of pplgG for seven days
30 postweaning was equal to or better than that of dietary zinc oxide in reducing diarrhoea
31 symptoms and in clearing faecal haemolytic bacteria for 14 days postweaning. These
32 observations warrant future trials of dietary pplgG in intensive swine production units to
33 establish its performance as an alternative to dietary antibiotics and zinc oxide for
34 preventing PWD.

35 **Keywords**

36 Postweaning diarrhoea; Immunoglobulins; IgG; Zinc oxide; Antibiotics; ETEC; *E. coli* F4;
37 Feed supplement; Faecal score

38

39 **1. Introduction**

40 After parturition, lactogenic immunity provides offspring with oro-gastric protection
41 against infectious pathogens (Hedegaard and Heegaard, 2016). In the conventional
42 swine production systems piglets are weaned at an immunologically immature age (3–4

43 weeks) depriving them of the continued supply of protective lactogenic antibodies at the
44 same time as they are placed in a new environment with increased risk of enteric
45 bacterial infections that may lead to postweaning diarrhoea (PWD) (Madec et al., 1998).
46 PWD is characterised by diarrhoea caused by enteric infection by Enterotoxigenic
47 *Escherichia coli* (ETEC) usually within three days postweaning (Fairbrother et al., 2005).
48 PWD, like other enteric infections in pigs, can be treated with dietary antibiotics
49 (primarily tetracyclines, penicillins and macrolides (Becker, 2010; DANMAP, 2015 ;
50 Wageningen University, 2012)), and/or zinc oxide (Pluske, 2013). Indeed, antibiotics and
51 zinc can improve average daily growth (ADG) in weaner piglets (Cromwell, 2002 ; Molist
52 et al., 2011), and dietary zinc can reduce frequency of PWD in weaner piglets (Owusu-
53 Asiedu et al., 2003 ; Pluske, 2013). The mechanisms by which zinc oxide increases ADG
54 and reduces diarrhoea in weaner piglets are not fully understood but seem to involve
55 both restoring plasma zinc levels to normal in piglets after weaning (Davin et al., 2013)
56 and improving intestinal homeostasis (Liu et al., 2014a; Liu et al., 2014b ; Shen et al.,
57 2014).

58 However, the widespread use of dietary zinc oxide in intensive animal production can
59 result in pollution of farmlands and groundwater through repeated fertilisation with
60 zinc-containing residual manure (Hill et al., 2005). In addition, zinc oxide in combination
61 with antibiotics appears to accelerate microbial antibiotic resistance by increasing the
62 rate of the exchange of antibiotic-resistance-gene containing plasmids in the microbiota
63 community in both soil and piglet intestines (Lin et al., 2016; Pal et al., 2015 ; Vahjen et
64 al., 2015); thus, there is a need to reduce both kinds of interventions.

65 As a sustainable and economically feasible alternative to antibiotics and zinc for treating
66 PWD, we have previously investigated the use of natural purified porcine

immunoglobulin G (pplgG) from pooled abattoir blood plasma (Hedegaard et al., 2016). First, it was established that natural pplgG does indeed contain immunoglobulin dependent anti-E. coli activity. Secondly it was shown that a dietary pplgG supplement in a model of PWD led to faster clearance of an ETEC challenge infection than seen in a comparable control group not provided with dietary pplgG (Hedegaard et al., 2016). This prompted us to further investigate this effect of pplgG in E.coli challenge models. A long term, low pplgG dose experiment and a short term, high pplgG dose experiment were performed. In both experiments dietary pplgG resulted in reduction of diarrhoea and in number of faecal haemolytic bacteria; moreover, in the second trial we observed that pplgG reduced diarrhoea and cleared the enteric infection faster than dietary zinc oxide. These observations warrant future experiments investigating the use of dietary pplgG postweaning as an alternative to dietary zinc oxide and antibiotics in treating/preventing PWD.

80

81 **2. Materials & methods**

82 The study comprised two infection experiments, both using E. coli O149 challenge at
83 two consecutive days post weaning. In experiment 1 (long term, low IgG dose pilot
84 study) 750 mg/day of pplgG was provided orally for five days before and 10 days post
85 weaning. In experiment 2 (short term, high IgG dose) 1.9 g of pplgG was given orally
86 twice daily for seven days after weaning/challenge. Experiment 2 also comprised a
87 group receiving dietary zinc oxide for 10 days after weaning.

88

89 **2.1. Purified porcine immunoglobulin G (pplgG)**

90 The pplgG was prepared from pooled pig plasma by expanded bed chromatography
91 (EBA) at Upfront Chromatography A/S (Copenhagen), as described previously
92 (Hedegaard et al., 2016). Concentrated porcine blood plasma was obtained from Daka
93 SARVAL A/S (Lunderskov, Denmark). The batch of pplgG used in first experiment
94 (experiment 1, see below) was the same as in (Hedegaard et al., 2016), whereas a new
95 batch of pplgG was prepared prior to the second (experiment 2, see below).

96

97 2.2. ELISA

98 The IgG concentration in the batches of pplgG was measured by a sandwich ELISA
99 (Hedegaard et al., manuscript in preparation), utilising a goat anti-pig IgG (Fc) antibody
100 (AAI41, Nordic Biosite ApS, Copenhagen) both for capture and detection. The IgG-
101 concentration of pplgG used in experiment 1 was 37.5 mg/ml and in experiment 2 the
102 pplgG concentration was 75 mg/ml.

103 Anti-E. coli activity was found in both batches of pplgG, used in this study, by indirect
104 whole-E. coli cell ELISA previously reported (Hedegaard et al., 2016); briefly 96 wells flat
105 bottom microtiter plates (Maxisorp, NUNC, Thermo Scientific, Denmark) were coated
106 with 100 µl fixed E. coli O138 (in-house strain isolated from piglet with diarrhoea) in 0.1
107 M sodium carbonate buffer pH 9.6 (OD₅₄₆ = 0.25) at 4 °C overnight. All subsequent
108 operations were performed at room temperature. Next day wells were washed four
109 times in PBS with 0.05% Tween 20 (PBS-T), and blocked with 200 µl PBS-T with 1%
110 bovine serum albumin (BSA; Sigma-Aldrich, Brøndby, Denmark) for 30 min when shaking
111 then followed by four times wash as above. The pplgG was added in 2-fold dilution
112 series (diluted in PBS-T 1% BSA from 10 to 0.02 mg/ml). After 1 h of incubation with
113 shaking and 4 washes in PBS-T, detection antibody HRP-conjugated goat anti-pig IgG

114 (GGHL-5P; ICL, SMS Gruppen; Rungsted, Denmark) diluted 1/2000 in PBS-T 1% BSA was
115 added and incubated for 1 h with shaking. After washing, plates were developed by TMB
116 Plus substrate (Kem-En-Tec, Taastrup, Denmark), 100 µl/well, and stopping colour
117 development by 100 µl/well 0.5 M H₂SO₄ (VWR—Bie & Berntsen A/S). Optical density
118 was measured by a Thermo Scientific Multiscan EX microplate reader at 450 nm
119 subtracting background absorbance at 650 nm.

120 Using the antigen specific ELISA it was found that the pplgG batch used in experiment 1
121 had lost 14% of activity in comparison to the original plasma pool, whereas the batch
122 used in experiment 2 had lost no activity (data not shown).

123

124 2.3. Experiments

125 2.3.1. Experimental procedure

126 Two separate factorial experiments at Aarhus University, Foulum, involving 12 ($10.8 \pm$
127 1.8 kg BW, from 2 litters) and 18 (7.3 ± 1.0 kg BW, from 2 litters) pigs for the first and
128 second experiments, respectively, were conducted (see Table 1). In both experiments,
129 piglets were weaned from sows that had been tested to be homozygote carriers of the
130 dominant gene encoding for intestinal F4 fimbriae receptors (Van Haeringen
131 Laboratorium, b.v., Wageningen, The Netherlands) on DNA extracted from hair sample.
132 Piglets were weaned from the sows at day 28 of age, and were challenged with *E. coli*
133 O149:F4 on two consecutive days (d 29 and 30 of age). Within each experiment, piglets
134 from different litters were equally distributed among treatments.

135 In the first experiment, half of the piglets (Table 1; Exp. 1, Group 1 + 2) received once
136 daily for 15 days (5 days preweaning and 10 days postweaning) 20 ml (750 mg) of pplgG,

137 provided by a 20 ml syringe; a small plastic tube was connected to the syringe and the
138 pplgG was slowly dipped in and piglets willingly lapped up, ensuring that no pplgG was
139 lost.

140 The other half of the pigs received 20 ml 0.9% NaCl (Table 1; Exp. 1, Group 3 + 4). Pigs
141 were provided the immunoglobulin product before feeding.

142 In the second experiment, pigs were allotted into three challenge-groups (Table 1; lower
143 part): two groups received no immunoglobulins but piglets were provided with 25 ml of
144 0.9% NaCl and provision of feed (from day of weaning) based on wheat, barley and
145 dehulled soybean, and with either 2500 ppm zinc oxide (Hammershøj Pharmacy,
146 Hammershøj, Denmark) for 14 days postweaning (Table 1; Exp. 2, Group 1), or no zinc
147 oxide (Table 1; Exp. 2, Group 2). The third group received pplgG via 20 ml syringe on the
148 morning before weaning (day 27), and on the morning of weaning (day 28). The pplgG
149 feeding was continued twice daily by drench gun for 7 days postweaning with a
150 provision of 25 ml (1.9 g) of pplgG twice daily (Table 1; Exp. 2, Group 3).

151

152 The animal experiment was conducted according to the personal license (Charlotte
153 Lauridsen, J. nr. 2012-15-2934-00125) obtained by the Danish Animal Experiments
154 Inspectorate, Ministry of Food, Agriculture and Fisheries, Danish Veterinary and Food
155 Administration, and animals were followed by proper veterinary surveillance throughout
156 the experiment.

157

158 2.3.2. Animals, feeding and housing

159 All piglets were tested susceptible to *E. coli* O149:F4 by using a DNA marker genotyping-
160 based test (van Haeringen laboratorium b.v., Wageningen, The Netherlands) on DNA
161 extracted from hair samples. From weaning and onwards, the pigs had ad libitum access
162 to feed and water. The feed was a standard diet for weaners prepared at the facilities at
163 Aarhus University, Foulum. Two littermates of similar weight were housed in pairs in
164 1.45 m × 1.7 m pens with concrete flooring and sawdust bedding. In experiment 1, the
165 unchallenged (Groups 1 + 3) and challenged pigs (Groups 2 + 4) were housed in different
166 rooms to avoid cross contamination with the challenge *E. coli* strain. A door separated
167 the rooms between the challenged and unchallenged piglets, and personnel changed
168 clothes/shoes before entering any of the rooms. Environmental conditions including
169 temperature (~24 °C), humidity (~50%) and bedding (sawdust) were similar in the two
170 rooms. Experiment 2 was conducted in the same rooms, but all pigs were challenged
171 with *E. coli*, and there was free passage between the rooms throughout the entire
172 experiment.

173

174 2.3.2.1. *E. coli* challenge

175 In both experiments, pigs were orally inoculated with 1.0×10^9 colony-forming units
176 (cfu) of *E. coli* O149:F4 in 5 ml 0.9% NaCl on day 1 and 2 after weaning (day 0 was the
177 day of weaning) using a syringe. After inoculation, the tube was flushed with
178 approximately 10 ml 10% NaHCO₃ in order to neutralize gastric acid and increase the
179 survival rate of the challenge strain in the stomach, and to ascertain that all the *E. coli*
180 suspension had been given to the piglets. The control pigs (exp. 1) received equivalent
181 amounts (approximately 5 ml) of 0.9% NaCl and 10 ml 10% NaHCO₃ in order to obtain

182 an equal level of stress associated with the oral inoculation as for the challenge
183 treatment.

184

185 2.3.3. Performance and diarrhoea assessment

186 Diarrhoea assessment was based on the consistency of the faeces (1 = hard, dry and
187 cloddy, 2 = firm, 3 = soft with shape, 4 = soft and liquid, 5 = watery and dark, 6 = watery
188 and yellow, 7 = foamy and yellow) from the day prior to challenge until 7 days after. A
189 faecal consistency score >3 was defined as a clinical sign of diarrhoea (Carstensen et al.,
190 2005). Before the E. coli challenge, and on day 2 after challenge, and daily until day 5
191 after challenge, and thereafter every second day during the second week after weaning,
192 faecal samples were collected from the rectum of the pigs and 1 g faeces was suspended
193 in a (1:10, wt/wt) peptone solution and homogenized by bag mixer (BagMixer100,
194 Interscience, St. Nom, France). Serial dilutions of the slurry were done prior to
195 enumeration of haemolytic E. coli on blood agar (BA; Oxoid) after aerobic incubation at
196 37 °C overnight. From each BA plate, five haemolytic E. coli colonies were selected and
197 tested for O149 and O138 type reactions by O-seroagglutination (Statens Serum Institut,
198 Copenhagen, Denmark). In addition, faecal samples were analysed for dry matter by
199 freeze-drying (ScanVac Coolsafe 55, Labogene Aps, Lynge, Denmark).

200 Feed intake was recorded daily for each pen and body weight of the pigs was recorded
201 at the beginning and weekly thereafter until the end of the experiment. Average daily
202 feed intake (ADFI) and gain (ADG) were determined based on pen by dividing the total
203 feed intake or total weight gain of pigs in each pen by days of feeding.

204

205 2.4. Statistics

206 The effects of pplgG, dietary zinc oxide or no treatment for seven days postweaning on
207 diarrhoea symptoms and bacterial count in both experiments were statistically analysed
208 using either Mann-Whitney test or Two-way ANOVA followed by Tukey's post-test, in
209 GraphPad Prism version 7.00 for Windows, GraphPad Software, La Jolla California USA,
210 www.graphpad.com.

211

212 3. Results and discussion

213 In this study we performed two infection experiments using an E. coli O149 challenge
214 strain at both day one and two after weaning. The administration of pplgG was different
215 in the two experiments: In experiment 1, 750 mg of pplgG was provided orally once daily
216 prophylactically for five days preweaning and then 10 days postweaning, whereas in
217 experiment 2, 1.9 g of pplgG was administered orally twice daily for seven days
218 postweaning. Two different pplgG batches were used for the two experiments, for
219 experiment 1 a 37.5 mg/ml batch and for experiment 2 a 75 mg/ml batch.

220

221 3.1. Disease

222 In both experiments diarrhoea was observed within one day after inoculation due to the
223 O149 ETEC challenge as observed by faecal scoring (Fig. 1A + B) and faecal dry matter
224 measurement (Fig. 1C + D). We observed in both experiments that the +pplgG
225 +challenge group (group 2 in exp. 1 and group 3 in exp. 2) had, at day five, significantly
226 less diarrhoea as compared to the control challenge groups (group 4 in exp. 1 and group

227 2 in exp. 2) that were not provided with pplgG as observed by a lower faecal scoring and
228 a higher percentage of faecal dry matter ($p < 0.03$; Fig. 1, days 1–5). In experiment 1 the
229 O149 ETEC challenge strain began to cease at day 7, however an unintended enteral
230 infection, starting around day 5–7 after weaning and lasting the remainder of the study
231 period was observed (Fig. 1A + C, days 5–21), coinciding with the appearance of an O138
232 ETEC strain in faeces (detected by PCR; data not shown). The presence of this
233 unintended infection somewhat obscured experiment 1, however the data from day 0 to
234 five were promising and prompted us to conduct a second experiment in which the daily
235 IgG dose was quadrupled but then only provided seven days post weaning. This batch of
236 pplgG had an IgG concentration of 75 mg/ml, and was administered twice daily,
237 amounting to a daily dose of 3.8 g.

238 Experiment 2 proceeded without any unintended infection, and group 3 (+pplgG
239 +challenge group) had significantly less diarrhoea than both group 1 and 2 (+zinc
240 +challenge and no treatment groups) within the first week after weaning ($p < 0.02$ Two-
241 way ANOVA; Fig. 1B + D); demonstrating that pplgG significantly reduced diarrhoea in
242 the PWD model within the first week post weaning to the same level or lower than
243 dietary zinc.

244 Moreover, our results indicate that it is not necessary to use pplgG prophylactically for
245 several days preweaning as there was a clear effect of pplgG in experiment 2 on
246 diarrhoea without the five days preweaning pplgG treatment applied in experiment 1
247 (Fig. 1). This also corroborates a previous study (Hedegaard et al., 2016) where pplgG
248 was mixed into the feed that was only available from weaning and decreased
249 diarrhoeagenic Enterobacteriaceae in comparison to a non-pplgG diet. Also, it has been
250 shown (Foged et al., 1986) that monoclonal anti-E. coli F4 fimbriae antibodies

251 administered at as well as after challenge, but not prophylactically, protected neonatal
252 piglets from an otherwise lethal challenge with F4+ ETEC. On the other hand, it seems to
253 be important to maintain a high administration frequency and an adequately high
254 antibody dose for limiting diarrhoea.

255

256 3.2. Microbiology

257 In both experiments pplgG helped clear the challenge strain within one week of
258 challenge (Fig. 2, days 1–7). However, as noted above, all groups in experiment 1
259 experienced an unintended O138 ETEC infection from day 4–9, as seen by an increase in
260 numbers of faecal haemolytic bacteria (Fig. 2A, days 4 + 7 + 9). Group 1 (+pplgG no
261 challenge) experienced this infection at day 4 while it was observed in group 2 (+pplgG
262 +challenge) on day 9. Thus, the daily administration of 750 mg of natural IgGs did not
263 provide protection against the unintended infection in neither of the two +pplgG groups
264 (Fig. 2A, groups 1 + 2, days 4–21), even though in vitro data support that pplgG binds to
265 O138 (and O149) ETEC and can inhibit their adhesion to intestinal epithelial cells in vitro
266 (Hedegaard et al., 2016). This might indicate that the unintended infection was
267 multifactorial, and/or that the daily dose of 750 mg natural pplgG used in this
268 experiment was not adequate to prevent this type of infection. Data from dose-
269 response field trials are needed before any further conclusions can be made on this
270 matter.

271 In experiment 2, only infection with the challenge-strain was observed. Confirming
272 previous results (Hedegaard et al., 2016), pplgG caused a faster clearance of
273 diarrhoeagenic (haemolytic) bacteria in group 3 than was observed in the other two
274 groups (Fig. 2B). Thus day 7 was the last day on which faecal haemolytic bacteria were

275 detected in group 3 (+pplgG), while these bacteria could still be detected on day 9 in
276 group 1 (+zinc group), and in group 2 (control) one piglet still had faecal haemolytic
277 bacteria on day 15 (Fig. 2B). Taken together, pplgG appears to intervene with the
278 colonization by the ETEC challenge strain shortening the period of infection significantly
279 (Fig. 2B, Day 5, $p < 0.02$) and was as efficient as dietary zinc in reducing the infection
280 with the ETEC challenge strain. Although this suggests that pplgG can shorten the
281 duration of diarrhoea in weaner piglets by decreasing the number of faecal
282 diarrhoeagenic haemolytic bacteria, this should ideally take place without a perturbing
283 the composition of the normal intestinal microbiota. In experiment 1, no change in the
284 faecal non-haemolytic bacteria were initially observed (Fig. 2C, days 1–5) however, as
285 the unintended infection emerged the number of faecal non-haemolytic bacteria began
286 decreasing (Fig. 2C, days 7–21). In group 2 (+pplgG +challenge) the faecal non-
287 haemolytic bacteria actually were not recovered for 3 days at the height of the
288 unintended infection (Fig. 2C, days 11–14), which coincided with the termination of
289 administration of pplgG. However in experiment 2, no changes in the count of faecal
290 non-haemolytic bacteria in any of the three other groups were observed (Figs. 2D),
291 indicating that pplgG does not intervene with the intestinal non-haemolytic commensal
292 microbiota and that the microbiota changes observed in experiment 1 was probably due
293 to the unintended infection. These observations are supported by preliminary next
294 generation sequencing data on the faecal microbiota composition of piglets fed pplgG
295 showing no change in non-haemolytic commensals (unpublished data).

296 Disease (diarrhoea) frequency, growth and feed conversion are primary end points for
297 swine producers however the two experiments described here comprised low numbers
298 of piglets ($n = 12/18$) and pens ($n = 4/3$) making it very difficult to analyse growth data
299 statistically. Also, the data on growth and feed intake showed a very large pig-to-pig

300 variation. For example, in experiment 2 half of the piglets in group 3 (+pplgG), for
301 unknown reasons, became anorexic during the third week resulting in almost no weight
302 gain for the group. Therefore it will be interesting to observe how pplgG
303 supplementation will influence disease resistance, growth and feed conversion in field
304 trials incorporating an adequate number of pigs to allow for appropriate statistical data
305 analysis to be performed. In spite of being a small preliminary study the results shown
306 here do however demonstrate the ability of dietary pplgG to clear an enteric ETEC
307 infection and thus pplgG could be used as an alternative to dietary zinc.

308

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454 **Tables**

455 Table 1: Study setup

Experiment 1	Group 1 +pplgG	Group 2 +pplgG+E.coli	Group 3 Control	Group 4 +E.coli
Number of piglets	3	3	3	3
<i>E. coli</i> F4+ challenge	No	Yes	No	Yes
750 mg pplgG (20 ml)	Yes	Yes	No	No
20 ml 0.9% NaCl solution	No	No	Yes	Yes
Avg. weight at weaning (kg)	11.2±3.1	10.6±1.7	10.4±1.9	11.0±1.2
Experiment 2	Group 1 + E.coli +Zn	Group 2 +E.coli	Group 3 +E.coli +pplgG	
Number of piglets	6	6	6 (5) ¹	
<i>E. coli</i> F4+ challenge	Yes	Yes	Yes	
2x1.9 g pplgG (2x25 ml)	No	No	Yes	
25 ml 0.9% NaCl solution	Yes	Yes	No	
Zinc oxide in feed	Yes	No	No	
Avg. weight at weaning (kg)	6.9±1.1	7.9±0.9	7.0±0.9	

456 ¹ One piglet was euthanized on day 3 post infection due to serious illness.

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459 **Figure legends**

460 **Figure 1. Faecal analysis:** Faecal diarrhoea score (**A+B**) and faecal dry matter (**C+D**); (●)
461 no treatment, no E. coli; (●) no treatment, +E. coli; (-■-) +pplgG, no E. coli; (▲)pplgG
462 treatment, +E. coli; (■) dietary zinc, +E. coli. Each data point is plotted and curves
463 outline the mean for each group. Vertical dotted line indicates the last day of pplgG
464 administration. Pairwise comparisons between groups on each day were tested for
465 statistical significance using Mann-Whitney test: a = IgG vs. Zinc ($p<0.03$); b = IgG vs.
466 Control ($p<0.03$); c = IgG vs. Zinc ($p<0.02$); d = IgG vs. Control ($p<0.01$); e = Zinc vs.
467 Control ($p<0.02$).

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470 **Figure 2. Faecal bacterial analysis:** Content (CFU/ml) of haemolytic bacteria (**A+B**) and
471 non-haemolytic bacteria (**C+D**). Symbols as for Figure 1. Each data point is plotted and
472 curves outline the mean for each group. Vertical dotted line indicates the last day of
473 pplgG administration. Pairwise comparisons between groups on each day were tested
474 for statistical significance using Mann-Whitney test: a = IgG vs. control ($p<0.02$).

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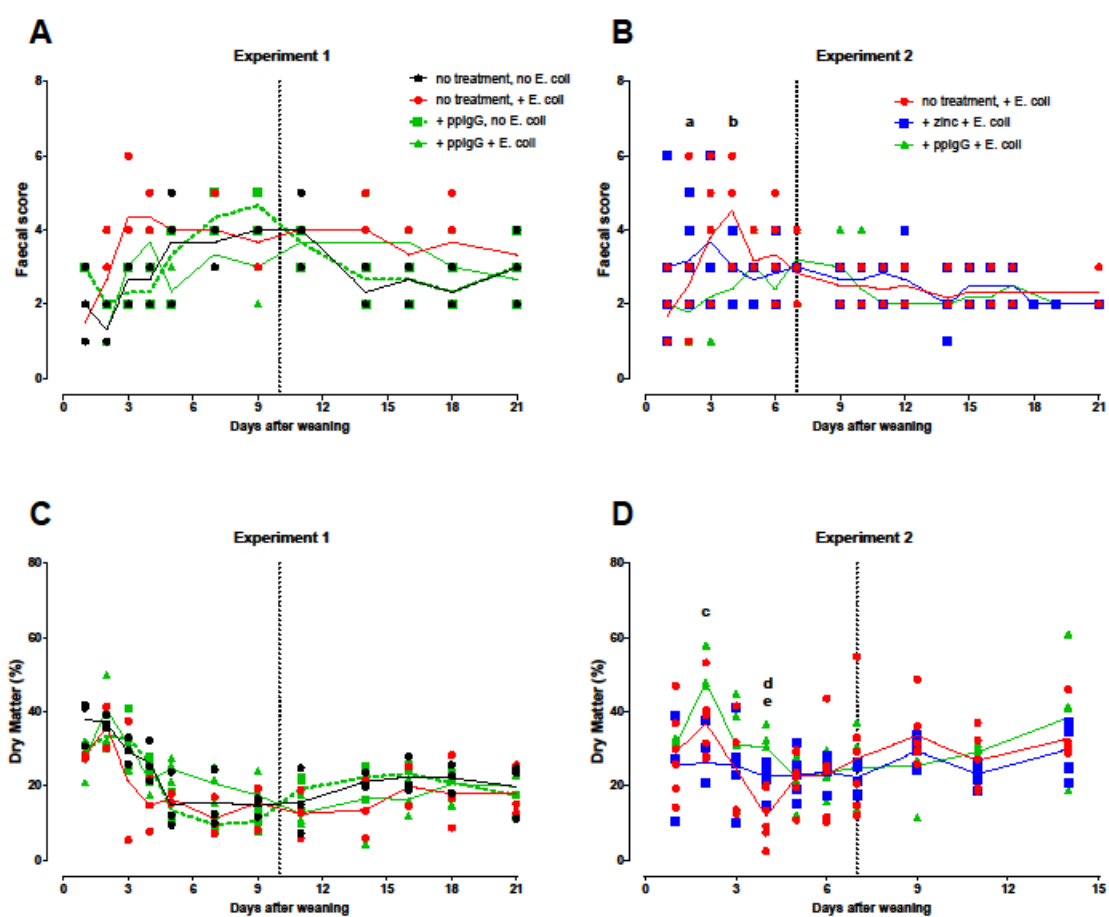
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480 **Figures**

481 **Figure 1**



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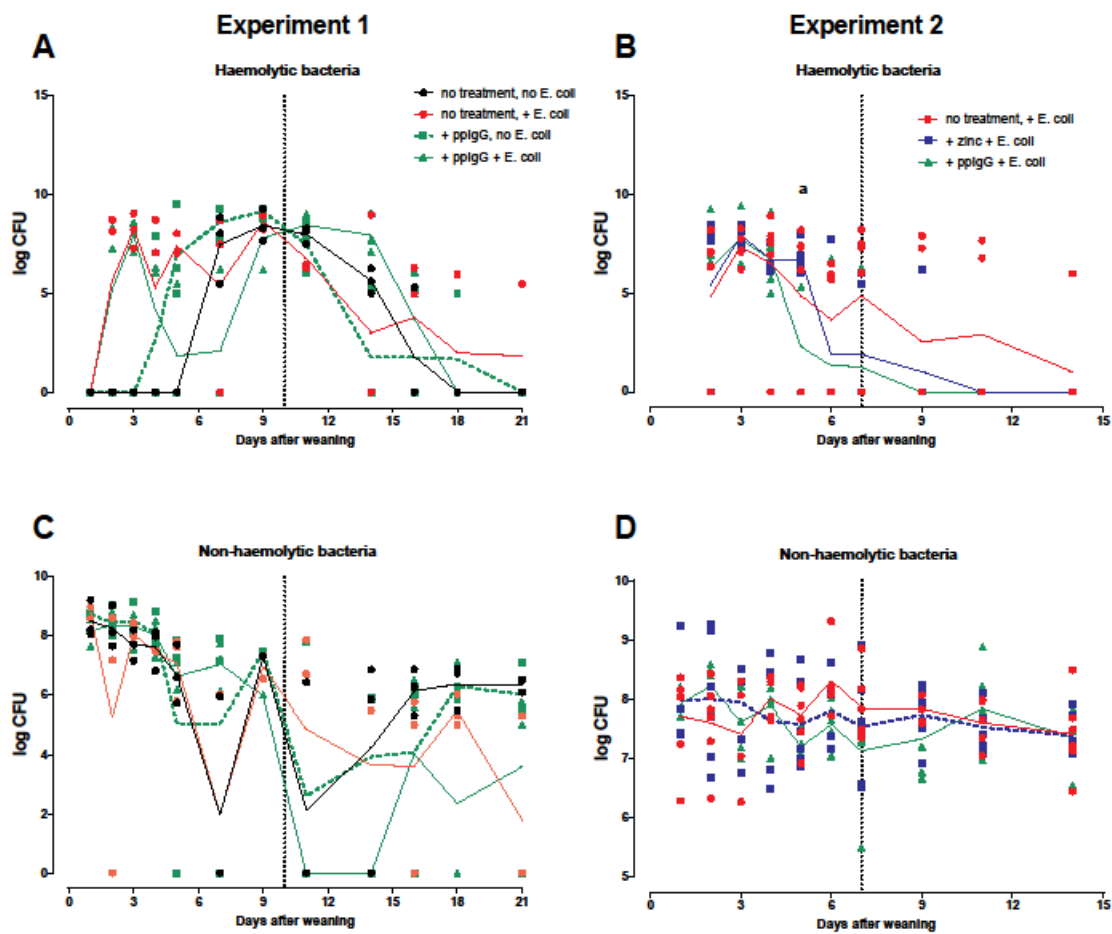
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490 Figure 2



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